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REVIEW ARTICLE

Quantitative image analysis for the characterization of microbial aggregates in biological wastewater treatment: a review

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Abstract Quantitative image analysis techniques have gained an undeniable role in several fields of research during the last decade. In the field of biological wastewater treatment (WWT) processes, several computer applications have been developed for monitoring microbial entities, either as individual cells or in different types of aggregates. New descriptors have been defined that are more reliable, objective, and useful than the subjective and time-consuming parameters classically used to monitor biological WWT processes. Examples of this application include the objective prediction of filamentous bulking, known to be one of the most problematic phenomena occurring in activated sludge technology. It also demonstrated its usefulness in classifying protozoa and metazoa populations. In high-rate anaerobic processes, based on granular sludge, aggregation times and fragmentation phenomena could be detected during critical events, e.g., toxic and organic overloads. Currently, the major efforts and needs are in the development of quantitative image analysis techniques focusing on its application coupled with stained samples, either by classical or fluorescent-based techniques. The use of quantitative morphological parameters in process control and online applications is also being investigated. This work reviews the major advances of quantitative image analysis applied to biological WWT processes.

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Abbreviations

2PLSM	Two-photon or multiphoton laser scanning microscopy
%Area	Total aggregates projected area distribution by equivalent diameter ranges
%Nb	Total number of aggregates distribution by equivalent diameter ranges
A	Aggregate area
a_L	Aggregates length
a_{Nb}	Number of aggregates
ANN	Artificial neural network
AR	Aspect ratio
A_{spec}<0.2 mm	Specific aggregate area for aggregates of D_{eq}<0.2 mm
A_{spec}≥0.2 mm	Specific aggregate area for aggregates of D_{eq}≥0.2 mm
B	Blue level of a pixel
BMP	Windows bitmap
CA	Cluster analysis
CARD-FISH	Catalysed reporter deposition fluorescence in situ hybridization
CCD	Charge Coupled Device
CF	Compactness factor
CLSM	Confocal Laser Scanning Microscopy
CMOS	Complementary Metal Oxide Semiconductor
COD	Chemical oxygen demand
Conv	Convexity
DA	Discriminant analysis
DAPI	4',6'-diamidino-2-phenylindole
D_{eq}	Aggregate Equivalent diameter
Ecc	Eccentricity
EF	Elongation factor

e_{Fil}	Filamentous fraction
e_{flocs}	Flocs fraction
$e_{microflocs}$	Microflocs fraction
EGSB	Expanded Granular Sludge Bed
EPS	Extracellular polymeric substances
Ext	Extent
FD	Fractal dimension
ferD	Feret diameter
FF	Form factor
FISH	Fluorescence in situ hybridization
F_{max}	Maximum feret diameter
fNb	Filaments number
freefNb	Free filaments number
GAO	Glycogen accumulating organisms
HCF	Heywood circularity factor
HSL	Hue saturation and lightness channels
JPEG	Joint photographers expert group format
LD	Load Disturbance
*L*fA	Total filament length per total aggregates projected area
L_{fi}	Total Filaments length per image
LSM	Laser scanning microscopy
L_{spec}	Specific total filament length
M1X, M1Y	First order moments
M2X, M2Y	Second order moments
MRI	Magnetic resonance imaging
N_{class}	Sum of any individual object/aggregate within a particular class
N_{obj}	Pixel sum of any individual object/aggregate
OLR	Organic Loading Rate
P	Aggregates perimeter
PAO	Phosphate accumulating organisms
P_{conv}	Convex envelope perimeter
PCA	Principal components analysis
PHA	Poly-β-hydroxyalcanoate
PHB	Poly-β-hydroxybutyrate
PLS	Partial least squares regression
PSD	Pore size distribution
R	Red level of a pixel
Rec%	Area recognition percentage
Rb	Robustness
Rg	Reduced radius of gyration
RGB	Red green and blue channels
Ro	Roundness
SAA	Specific Acetoclastic Activity
SBR	Sequencing batch reactor
SDS	Sodium Dodecyl Sulfate
Sol	Solidity
SRT	Sludge retention time
SS	Suspended Solids
SVI	Sludge volume index
TA	Total aggregates area
TIFF	Tagged image file format
TL	Total filaments length
TL/TA	Ratio between total filaments length and total aggregates area
TL/TSS	Total filament length per total suspended solids
TL/VSS	Total filament length per volatile suspended solids
TSS	Total suspended solids
TV	Total particles volume
UASB	Up-flow anaerobic sludge blanket
UFBR	Up flow anaerobic fixed bed reactor
VSS	Volatile suspended solids
VSS/TA	Volatile suspended solids per total aggregates projected area
W	Aggregates width
WWT	Wastewater treatment
WWTP	Wastewater treatment plant
$X_{biomass}$	Sludge concentration
X_{ni}, y_{ni}	Coordinates of each objects pixels

Introduction

Human vision is superb, but qualitative and subjective (since the results depend on the observer). Computers can do better than humans at extracting quantitative information and may reduce the subjectivity of image interpretation. Image analysis can be seen as the simple extraction of information from from pictures (Glasbey and Horgan 1995). These pictures are the sum of a vast number of tiny points (pixels), which, after computer processing and analysis, allow the retrieval of rigorous morphological quantitative information. The ever decreasing costs of computational data processing open a window of new methodologies that can be explored and applied in microbial processes.

In biotechnology, and particularly in WWT processes, the tedious, subjective, and time-consuming techniques used in traditional classification and quantification of microorganisms are being replaced by automated and semiautomated quantitative image analysis techniques based on digital technology. The advantages of image processing over traditional methods include applicability and usefulness, automated or semiautomated, long-term cost, operator independent, objectivity, quantitative, reliability and accuracy, and speed. However, also some disadvantages may be found, namely, lack of implementation on full-scale installations, initial cost, trained personnel, complexity, and distinction of live and active organisms.

Image analysis, in general, refers not only to the strict image analysis process but also to the previous processes of

image capture and processing (Dougherty, 1994). The overall process can be summarized in three main steps:

1. Sample preparation and image acquisition: The object to be analyzed should be carefully sampled and prepared according to the study objective. Most often a wet mount is used (diluted if necessary), but pretreatment such as staining can also be employed. An image is then captured with the help of a digital camera coupled to a microscope. This can be a stereo, optical, fluorescence, confocal laser scanning (Lopez et al. 2005), or electron microscope (Singh and Viraraghavan 2003). The images are saved on magnetic or optical data carriers using specialized software. The development of automated processes for stained images is gathering great momentum nowadays (Pandolfi et al. 2007; Neu et al. 2010; Mesquita et al. 2011a, 2013).
2. Image processing: The set of operations performed to transform a raw input image into a final output image allowing the analysis of the objects of interest is called image processing (Dougherty, 1994). One of its objectives is to improve the quality of the images by background determination, noise reduction, debris elimination, and object enhancement. The first step usually focuses on the determination and removal of background light differences. The use of frequency and space-domain filtering by means of linear and nonlinear filters reduces noise. Object enhancement can be accomplished by the use of morphological or histogram based operations. The image may be then subjected to a segmentation process, and, as a result, a labeled or binary image is obtained, containing the information required for a given application. A further postprocessing step, including shape and size operations, connectivity, and texture operations, may also be necessary to eliminate debris. Erosion, dilation, opening, closing, and distance transforms are included in the shape and size operations group, while skeletonisation, thickening, pruning, and watershed are examples of connectivity operations (Glasbey and Horgan 1995).
3. Image analysis: Finally, the morphological parameters are analyzed. Usually, the objective of image analysis concerns the determination of descriptors describing the morphology, i.e., the size and shape of objects; the composition of objects, i.e., exploration of their internal structure, for example distribution of microbes, identification of microbial species and spatial arrangement of microorganisms (Liwarska-Bizukojc 2005).

Activated sludge and anaerobic digestion processes are the dominant biological processes in WWT; therefore, the main focus in quantitative image analysis development is targeted on monitoring and control of these two processes for optimization purposes.

The aim of this paper is to gather the most relevant work currently being developed on the application of quantitative image analysis techniques in biotechnology and especially in biological WWT. The main steps involved in image acquisition, processing, and analysis, from sampling to data analysis, are briefly presented in "Fundamentals on quantitative image analysis protocol." The main achievements along with current approaches ("Fields of application") and future trends ("Current state synopsis and future trends") will be discussed.

Fundamentals on quantitative image analysis protocol

Digital images are pictures that have been converted into a computer-readable binary format consisting of logical 0's and 1's. Usually, an image refers to a still picture that does not change with time. The frequency with which information is transmitted, stored, processed, and displayed is increasing rapidly, and thus, the design of engineering methods for efficiently improve and/or employ this technology in diverse fields is of great interest. The main steps to consider in a quantitative image analysis protocol are shown schematically in Fig. 1, while the main parameters obtained with quantitative image analysis are presented in table 1.

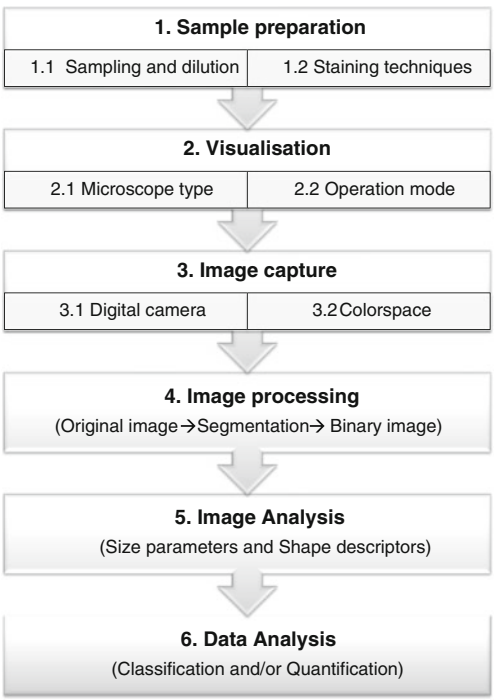

Fig. 1 Main steps in quantitative image analysis of biological processes. Each step includes the main aspects to be considered in the protocol

Table 1 Key parameters obtained by quantitative image analysis used for biological wastewater treatment monitoring and control

Name	Formula	Reference
Area recognition percentage	$\mathrm{Rec}\% = \frac{\sum_{i=1}^{n_{\mathrm{obj}}} A_i}{\mathrm{TA}}$	Amaral 2003
Aspect ratio	$\mathrm{AR} = 1 + \frac{4(\mathrm{lenght-width})}{\pi\ \mathrm{width}}$	Grijspeerdt and Verstraete 1997
Compactness	$\mathrm{CF} = \frac{\sqrt{\frac{4}{\pi}A}}{F_{\max}}$	Russ 1995
Eccentricity	$\mathrm{Ecc} = \frac{(4\pi)^2\left(M_{2x}-M_{2y}\right)^2+4M_{2xy}^2}{A^2}$	Glasbey and Horgan 1995
	$M_{2xy} = \frac{1}{A}\sum_{n=1}^{N}\left(x_n^i - M_{1x}\right)\left(y_n^i - M_{1y}\right)$	
Equivalent diameter	$D_{\mathrm{eq}} = \sqrt{\frac{\mathrm{Area}}{\pi}}$	Russ 1995
Form factor	$\mathrm{FF} = \frac{4\pi\ \mathrm{Area}}{\mathrm{Perimeter}^2}$	Grijspeerdt and Verstraete 1997
Fraction of microflocs	$e_{\mathrm{microflocs}} = \frac{\mathrm{Area}_{(\mathrm{microflocs})}}{\mathrm{Area}_{(\mathrm{flocs+microflocs+filaments})}}$	Heine et al. 2002
Percentage area of aggregates within a size class	$\%\mathrm{Area} = \frac{\sum_{i=1}^{n_{\mathrm{class}}} A_i}{\mathrm{TA}}$	Amaral 2003
Reduced radius of gyration	$\mathrm{Rg} = 2\frac{\sqrt{M_{2x}+M_{2y}}}{D_{\mathrm{eq}}}$	Pons and Vivier 1999
	$M_{1x} = \frac{1}{A}\sum_{i=1}^{N} x_n^i$	
	$M_{1y} = \frac{1}{A}\sum_{i=1}^{N} y_n^i$	
	$M_{2x} = \frac{1}{A}\sum_{i=1}^{N}\left(x_n^i - M_{1x}\right)^2$	
	$M_{2y} = \frac{1}{A}\sum_{i=1}^{N}\left(y_n^i - M_{1y}\right)^2$	
Roundness	$\mathrm{Ro} = \frac{4}{\pi}\ \frac{\mathrm{Area}}{P_{\mathrm{conv}}^2}$	Glasbey and Horgan 1995
Shape factor	$\mathrm{ShF} = \frac{\mathrm{Perimeter}^2}{4\pi\ \mathrm{Area}}$	Noesis 1998
Total filament length per total aggregates projected area	$L\mathrm{fA} = \frac{L_{\mathrm{spec}}}{A_{\mathrm{spec}<0.2\mathrm{mm}}+A_{\mathrm{spec}\geq 0.2\mathrm{mm}}}$	Araya-Kroff et al. 2004
Total filaments length per volatile suspended solids	$\mathrm{TL/VSS} = \frac{L_{\mathrm{spec}}}{\mathrm{VSS}}$	Amaral 2003
Total filaments length per total suspended solids	$\mathrm{TL/TSS} = \frac{L_{\mathrm{spec}}}{\mathrm{TSS}}$	Amaral 2003
Volatile suspended solids per total aggregates projected area	$\mathrm{VSS/TA} = \frac{\mathrm{VSS}}{A_{\mathrm{spec}<0.2\mathrm{mm}}+A_{\mathrm{spec}\geq 0.2\mathrm{mm}}}$	Araya-Kroff et al. 2004

Sample preparation

Sampling and dilution

The first step is to take a representative and homogenous sample of the object (microorganism, floc, aggregate, biofilm, etc.) under study using a special device that does not damage the sample integrity. This is an important and difficult task, especially when applied online, since microorganisms and aggregates can be very fragile. To facilitate the scale-up to monitor real WWTP, the correct and homogenous sampling of a representative sample of the entire plant is a critical step that should be optimized. However, few attentions are being given to this step.

When using wet-mount procedures, the liquid layer thickness must allow high magnifications, focusing on a

thin plane (Grijspeerdt and Verstraete, 1997). Another factor needing attention is dilution. Usually, biomass samples must be prepared for image analysis using an optimized dilution factor. A considerable number of WWT processes operate with high biomass contents, suggesting that, during image acquisition, samples should be diluted for an adequate characterization. Therefore, dilutions aid microscopic inspection of sludge, and when using image analysis methodologies, they allow the system to be evaluated accurately (Mesquita et al., 2010b). When the dilution is excessive, the observer may unconsciously search objects, overestimating them. Thus, dilution techniques can be problematic as the amount of screened biomass decreases and they could lead to morphological changes of the aggregated biomass. An example can be seen in Fig. 2a and d where a big floc was carefully placed in the center of the image, and in this way will not be cut off by the boundaries and consequently eliminated. If the dilution is insufficient, the objects will be overlaid and thus underestimated (Fig. 2b, e). Regarding the study of da Motta et al. (2002b), it was found that when samples are not diluted, aggregates and filaments may appear superimposed on images, which can be misleading. The optimal dilution value is determined as the lowest dilution that enables the maximum percentage of objects to be recognized. The area recognition percentage (Rec%; Table 1) is the ratio between the area of objects that are completely inside the image and the total area of objects (TA) in the image, including those that are cut off by the image boundaries and are discarded from the aggregates characterization (Amaral 2003; Fig. 2c, f).

When working with granular sludge, it is necessary to distinguish between micro- and macroaggregates because of the large differences in sizes and consequent problems in image acquisition. Usually microaggregates are considered as the objects with equivalent diameter (D_{eq}; Table 1) <0.2 mm, and macroaggregates or granules as the ones with $D_{eq} \geq 0.2$ mm (Araya-Kroff et al. 2004). For the acquisition of filaments and microaggregate images, a volume of 35 μL from the diluted sample is distributed on a slide and covered with a 20×20 mm coverslip for visualization and image acquisition. Image acquisition is obtained by dividing the coverslip into 42 identical fields and taking a photo of each imaginary square (Costa 2008). Concerning to macroaggregate images, an arbitrary volume is transferred to a Petri dish for visualization and image acquisition. All the aggregates present in this volume are digitalized. The VSS content in the Petri dish is measured to standardize the measurements in each sample by its VSS (Costa 2008).

According to Costa et al. (2007, 2009a, b), the optimal dilution factor is between 1:5 and 1:10 for anaerobic granular sludge samples with volatile solids around 30 g L^{-1}. Regarding aerobic activated sludge (Mesquita et al. 2010b), an optimum 1:5 dilution factor was found for samples with total suspended solids (TSS) ranging from 2.3 to 4.7 mg L^{-1}.

Staining techniques

Although most of the literature on image analysis applications on biological WWT processes describes procedures for unstained samples, coupling it with dyes and molecular techniques, such as staining or fluorescence in situ hybridization (FISH), is expanding. This technique allows rapid quantification of several microorganisms (Hug et al. 2005) and deeply characterization of microbial aggregates (Liu et al. 2010). The combination of fluorescence staining techniques, confocal laser scanning microscopy (CLSM), two-photon or multiphoton laser scanning microscopy (2PLSM), intensity imaging and lifetime imaging offers a wealth of opportunities in order to collect multiple pieces of information from highly complex biological systems such as microbial aggregates and biofilms (Neu et al. 2010).

In WWT processes, the use of a light microscope is a very common way to characterize the state of the sludge. However, the use of this equipment can also lead to some failures in microorganisms' characterization, regarding specific microbial communities, which are hardly identified. Thus, the classical Gram and Neisser stains are routinely used in floc and filamentous organism characterization (Jenkins et al., 2003). However, in terms of filamentous organism identification, the Gram staining method is the most widely used staining technique where a differentiation between positive and/or negative status is performed based on the chemical and physical properties of the cell wall. This procedure was succesfully applied by Wagner et al. (1994) and Bradford et al. (1996) and combined with FISH probes for the identification of a specific number of filamentous organisms. The Neisser staining is also used to differenciate filamentous organisms with morphological similarities. However, it is also very usefull for polyphosphate intracellular granules detection in phosphorus removal systems (Eikelboom 2000; Jenkins et al. 2003; Serafim et al. 2002). Both staining procedures are carried out on biomass smears fixed on glass microscopic slides and observed by optical microscopic observation (at ×100 magnification). Thus, in Gram stain, filamentous organism differentiation are observed as totally colored (red is negative or blue violet is positive; Jenkins et al. 2003). In the case of Neisser stain for intracellular storage compounds, only polyphosphate granules inside the cells appear colored (positive if intracellular granules are blue violet) (Serafim et al. 2002).

An excellent way to overcome some of the problems of studying microbial populations without resorting to traditional methodology is to use fluorescent probes. These are short sequences of DNA (16–20 nucleotides) labeled with a fluorescent dye. These sequences recognize 16S ribosomal ribonucleic acid (rRNA) sequences in fixed cells and hybridize with them in situ (Sanz and Köchling 2007). FISH has been frequently used to determine the contents of specific microorganisms in sludge samples and typically to identify

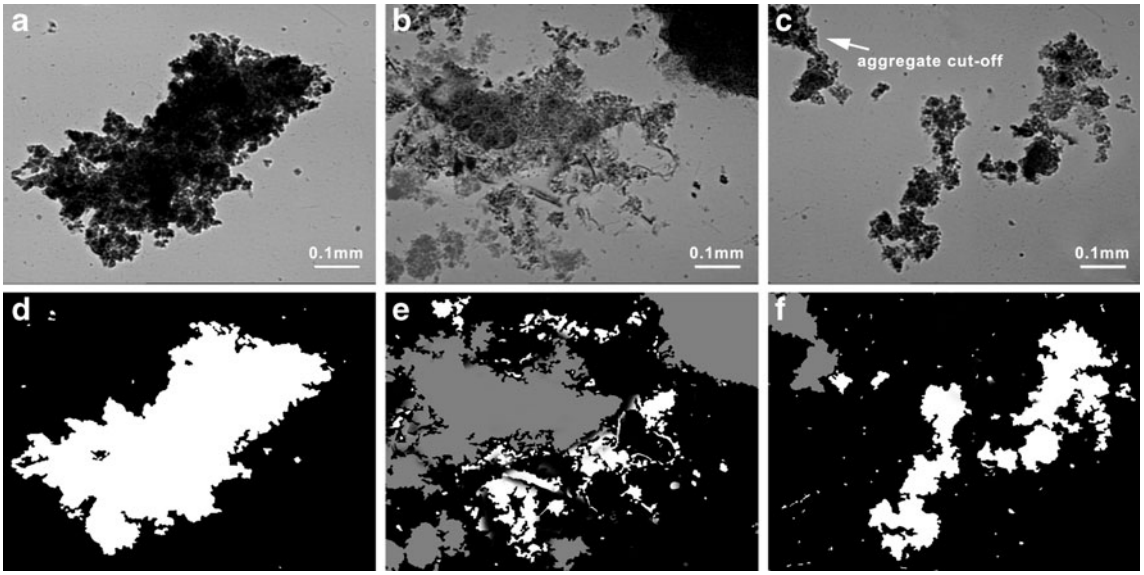

Fig. 2 Bright field images (**a**–**c**) and respective binary images (**d**–**f**) from micro-aggregates with excessive dilution (overestimation) (**a**, **d**), insufficient dilution (underestimation) (**b**, **e**), and ideal dilution (**c**, **d**). The flocs cut-off by the boundaries (in *gray*) are discarded during image analysis. The aggregates considered for sample characterization are represented in *white*

filamentous organisms which are responsible of bulking and foaming conditions in WWT processes (Wagner et al. 1994, 1996; de los Reyes et al. 1997; Erhart et al. 1997; Schuppler et al. 1998a, b; Davenport et al. 2000; Kanagawa et al. 2000; Wagner et al. 2002; Lopez et al. 2005; Hug et al. 2005; Blackall et al. 1996; Rossetti et al. 1997, 2005; Blackall et al. 2000; Liu et al. 2001a, b; Seviour et al. 2006; Serafim et al. 2002; Carvalho et al. 2007). It is stated that FISH improves biological processes; however, several disadvantages were pointed out by Sanz and Köchling (2007) such as the previous knowledge of the domain microbial population. It should be kept in mind, however, that the difficulty in quantifying the hybridization intensity remains a barrier to overcome in the FISH protocol. Further studies should be performed to validate the usefulness of this technique coupled to stained samples. Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) coupled with CLSM can be used to significantly enhance the signal intensities of hybridized cells, although is rather expensive and requires enzymatic pretreatment. Currently, it has been applied to improve the retrieval of information from biofilms without the destruction of the biofilm structure (Amann et al. 1998; Lupini et al. 2011), and it has been also found the usefulness of CARD-FISH to heterotrophic bacteria in marine samples and soil (Pernthaler et al. 2002; Schmidt et al. 2012).

Using other fluorescent nucleic binding dyes, the Live/Dead BacLight bacterial viability kit is an available mixture of SYTO 9 green- and a red-fluorescent nucleic acid stain, propidium iodide (PI). Through microscopic examination, viable bacteria are stained by SYTO 9 and damaged bacteria are stained by PI (Invitrogen Molecular Probes 2004). Gram status can also be performed through the use of fluorescent nucleic acid binding dyes. The Live Baclight bacterial Gram stain kit allows bacterial classification as Gram positive or Gram negative without the use of fixatives. This kit utilizes a mixture of SYTO 9 green- and a red-fluorescent nucleic acid stain, hexidium iodide (HI). Gram-negative bacteria are stained by SYTO 9, and Gram-positive bacteria are stained by HI (Invitrogen Molecular Probes 2011).

Visualization

Microscope type

The first effective step in an automated or semiautomated image analysis protocol consists of the visualization of objects. Since most techniques use grayscaled images, optical (or light) microscopes are the most widely used to visualize samples. Standard bright-field microscope images of single or multiple cells typically contain dark objects against a lighter background, making them appropriate for most applications of quantitative image analysis. Phase contrast lenses create a halo surrounding the objects that can give some problems in subsequent processing steps (Pons and Vivier 1999). However, they can be suitable and advantageous for measuring filamentous microorganisms. Recently, Mesquita et al. (2010a) showed a comparison between bright field and phase contrast image analysis techniques in activated sludge morphological characterization. They found that it was feasible to acquire both filaments and flocs in a single image through bright field microscopy (Mesquita et al. 2010a; Fig. 3). However, most techniques use bright field images for floc characterization

and phase contrast images for filament characterization, which doubles the procedure time and effort. Therefore, techniques that minimize costs and time spent with the analysis should be favored.

Recently, new advanced techniques have been established including laser scanning microscopy (LSM), magnetic resonance imaging (MRI), and scanning transmission X-ray microscopy. These new techniques allow in situ analysis of the structure, composition, processes, and dynamics of microbial communities (Neu et al., 2010). The use of image analysis combined with epifluorescence techniques is usually followed by fluorescence microscopy or CLSM (Kuehn et al. 1998; Schmid et al. 2003). CLSM is particularly adapted to the study of 3D structures, such as aggregates of cells (Lawrence et al. 1998) and aerobic granules (Liu et al. 2010). According to Lopez et al. (2005), selection of the most appropriate technique depends on the object being investigated. For flocs with high cell density, the use of 2PLSM is preferred, since it provides a clear image of the internal structure of the aggregate. For this purpose, epifluorescence microscopy gave unreliable quantification of red stained cells in dense aggregates, and CLSM did not adequately represent the internal filamentous structure and the location of stained cells within dense flocs. However, for typical activated sludge flocs and procedures, epifluorescence and CLSM have proved to be adequate (Lopez et al. 2005).

Operation mode

Most of the actual routine image analysis applications on microorganisms is run offline and operator assisted for manual sampling in bioreactor, slide preparation (including staining), and image capture (Pons and Vivier 1999). Some automatic online systems have already been reported (Govoreanu et al. 2002; Jenné et al. 2002). These systems involve circulation loops to bring the sample to the microscope. The major problem with online systems is the limited depth of focus, so the cells need to be kept close to the focus plane by forcing them through a capillary or flow cell (Maruhashi et al. 1994; Yu et al. 2005). Galindo et al. (2005) described an image analysis technique for the in situ characterization of dispersions occurring in bioreactors. However, this technique is very difficult to implement widely, due to equipment costs and poor image quality, which is usually blurred. He et al. (2012) developed a promising non intrusive optical sampling technique to obtain digital images of particles. It consists of an automated stroboscopic lamp placed in opposite to the digital camera to backlight suspended particles in a tank and produce shadows of particles. It is possible that the advances in digital cameras, especially regarding shutter speed, may enable the acquisition of images with adequate quality to use quantitative image analysis online in the near future. When the problem of stability and integrity of the samples for online systems is overcome, the emergence of new online techniques, capable of capture sharp and precise images, may represent a milestone in increasing the use of quantitative image analysis techniques for biological processes monitoring. However, an increase in the equipment costs must be considered.

Image capture

Digital camera

With the development of digital cameras in the last few years, this step in image analysis protocol is easily applicable. An image is captured by a digital camera and recorded by a sensor. Most used are charge coupled device (CCD) and complementary metal oxide semiconductor (CMOS) sensors. Currently, CMOS sensors can work with higher frame rates but are affected with higher noise levels than CCD sensors. Nevertheless, CCDs dominate much of the camera market because they are lightweight and cheaper (Nixon and Aguado 2002).

Color space

The intensity of the transmitted or emitted light by the surface element corresponding to the pixel on the initial analog image is nowadays transformed into a number coded on 8 bits for monochrome images (the most widely used), corresponding to the 256 gray or brightness levels that range from black (0) to white (255). In fluorescence images, the color is an important factor, and the images are usually recorded in 24 (3×8) bits. Most used color space is the RGB (red, green and blue channels), followed by the HSL (hue, saturation, and lightness channels). After the capture step, the image must be stored. At the moment the resolution can go up to 21 Mpixels (~63 Mbytes); because of disc space and processing time constraints, however, the use of smaller and uncompressed images is usually sufficient and preferred.

Image processing

Subsequent to acquisition, images are processed to obtain a final image, either greyscale, labeled, or binary, containing the information required for a given application. The first step in any processing method normally focuses on the determination and removal of the background or background light differences (Amaral 2003).

Numerous programs for the automatic image processing became available in the last years. Amaral (2003) reported three specialized automated programs for filaments, flocs, and granules isolation and quantification. The main stages of these programs are:

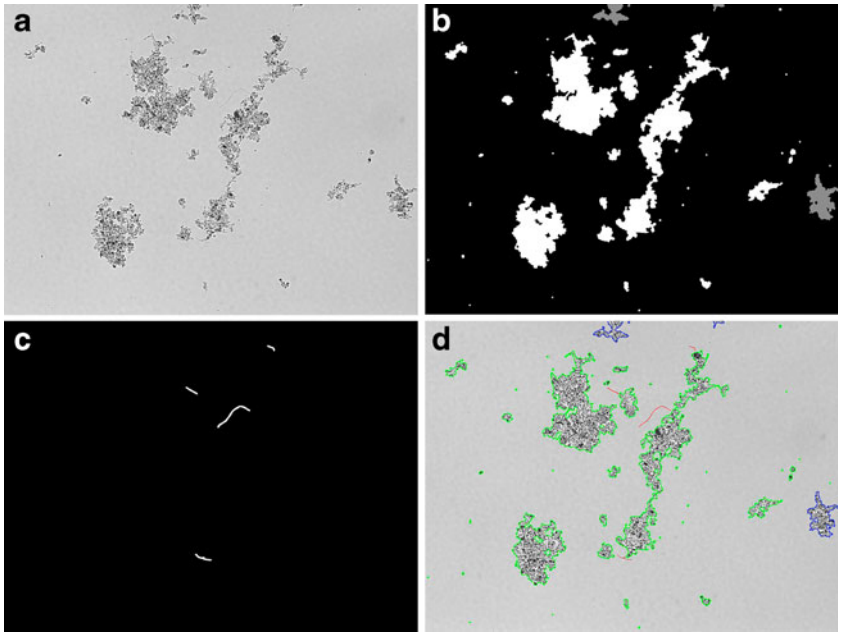

Fig. 3 Bright-field image processing: **a** original image from a lab-scale activated sludge system acquired in a Olympus BX51 optical microscope (Olympus, Tokyo, Japan) with ×100 total magnification, **b** aggregates binary image after the segmentation step, **c** filaments binary image after the segmentation step, and **d** labeled image showing the definition of aggregates in *green* and filaments in *red*

- Changeable processing definition: apart from the standard built in processing definitions, the operator may redefine a series of processing parameters regarding the background identification protocol, segmentation threshold, and object and debris identification.
- Pretreatment: resides in the gray image improvement through background correction and contrast enhancement between objects and background.
- Segmentation: this step transforms the greyscale image into a binary image, with pure white objects (1's) and pure black background (0's).
- Posttreatment: uses morphological operations such as erosion and reconstruction and parameters to identify and delete small debris, fulfill gaps inside objects, and remove boundary cut-off objects.
- Labeling: attributes a discrete number to each pixel belonging to a specific aggregate that differs from aggregate to aggregate.

Other procedures (Table 2) may be found in studies reporting activated sludge characterization (Jenné et al. 2006) and semiautomatic identification of protozoa and metazoa (Amaral 2003; Perez et al. 2006). Liu et al. (2001a, b) developed a program for the analysis of bacteria morphotypes in microbial communities, and Daims et al. (2006) developed a computer program that incorporates digital image analysis and 3D visualization functions from epifluorescence microscopes and CLSM.

A critical step is the filament identification. The reduced radius of gyration (Rg; Table 1) is a global descriptor useful to discriminate large globular features (debris or pellets) from filamentous objects. According to Pons and Vivier (1999), an Rg of 0.7 represents a disc and 0.84 represents a filament. Another strategy to distinguish filaments from flocs was introduced by Contreras et al. (2004), who studied competition between filamentous and nonfilamentous bacteria by image analysis using two different parameters, roundness (Ro; Table 1) and Rg. They concluded that objects with a Ro<0.38 or a Rg>1.06 should be considered filamentous microorganisms, although Rg was more accurate than Ro.

Image analysis

Image analysis generally allows the determination of morphological, physiological, and fractal dimension parameters of the detected objects in the final binary or labeled image. The type of parameters collected during the image analysis step depends on the type of final image (binary, labeled, or grayscale) and on the required data for each specific application.

Usually, the morphological data obtained can be divided in size parameters and shape descriptors. The first are obtained by automatically counting the pixels belonging to each object, and expressing its size. The number, area, D_{eq}, and length of an object belong to this group. Relatively to shape descriptors, it refers to morphological parameters that describe the form of the object, including its circularity, elongation, shape factor, roundness, compactness, eccentricity (Table 1), etc. (Amaral 2003; Pons and Vivier 1999).

Data analysis

Data analysis refers to the direct application of the parameters obtained with quantitative image analysis in a wide range of applications. It can be used for classification of the operation status of WWTP or detection of microorganisms,

Table 2 Overview of key works describing quantitative image analysis techniques and processing

References	Microscope	Total magnification	Illumination	Acquisition (images)	Software	Processing
Grijspeerdt and Verstraete 1997	Stereo	×50	Dark field	CCD camera (±10)	Microsoft Visual C++	(1) Segmentation (a threshold has to be defined in order to distinguish objects from the background—simply a lower and upper limit of intensity)
Amaral et al. 1999	Optical light	×400	Phase contrast	CCD camera (–)	Visilog 5.1	(1) Image enhancement (2) Recognition—Protozoa (thresholding using a manual threshold) (3) Image treatment (elimination of small flocs, etc.) (4) Characterization and quantification
da Motta et al. 2001b	Optical light	×100	Bright field	Video camera (70)	VisilogTM 5	(1) Image enhancement (2) Segmentation (considers the variance of the gray-level histogram) (3) Recognition—discrimination between flocs and small debris and filamentous bacteria (based on the size and the gyration radius—objects with a projected area larger than 200 pixels and a reduced gyration radius larger than 1 are filaments)
Jenné et al. 2002	Optical light	×100	Phase contrast	CCD camera (50)	Matlab Image Processing Toolbox	(1) Segmentation (novel histogram-based thresholding algorithm) (2) Recognition—distinction between flocs and filaments (using the reduced radius of gyration, with a discriminating value of 1.1. A second criterion was added to separate filaments touching a floc: objects with less than 30 % pixels that are lighter than the background are considered to be filaments) (3) Characterization and quantification
Contreras et al. 2004	Optical light	×1,000	Phase Contrast	CCD camera (±12)	Global Lab Image 2.10	(1) Segmentation (critical threshold was calculated as the grey level value that corresponded to the maximum of the gray level histogram second derivative) (2) Characterization and quantification
Araya-Kroff et al. 2004	Optical light (filaments), stereo (aggregates)	×100 (filaments), ×100, (microaggregates), ×40 (macroaggregates)	Phase Contrast (filaments) Bright Field (aggregates)	CCD camera (±100)	Matlab	Three programmes (filaments, microaggregates and macro-aggregates)
Pandolfi and Pons 2004	Optical light	×100	Bright Field	Video camera (70)	VisilogTM 5	(1) In the first part the colour system of the Gram image is changed from RGB to HIS (2) Image enhancement (3) Segmentation (threshold algorithm)

Table 2 (continued)

References	Microscope	Total magnification	Illumination	Acquisition (images)	Software	Processing
						(4) Recognition (Size and Rg) (5) The Gram characteristics of each of the remaining fragments are finally evaluated. A pixel is considered to be blue when its level (B) on the blue primitive of image ARGB is higher than its level on the red primitive (R). Inversely a pixel is considered to be red when R>B (6) Two ways of analysing the results from the image analysis procedure were tested: a binary method, a fragment is considered to be Gram positive (respectively Gram negative) if more than 50 % of its pixels are blue (respectively red); a fuzzy method, with which it is recognized that the decision between blue and red might (respectively Gram negative) if more than 66 % of its pixels are blue (respectively red). When the percentage of blue pixels is in the range 33–66 %, the fragment is said to be Gram undetermined
Yu et al. 2009	Micro lens	–	Halogen illuminator	CCD camera (40)	NI Vision Assistant	(1) Image enhancement (2) Segmentation (entropy, binarization) (3) Characterization and quantification Note: A flow cell with magnetic pump was used to provide continuous samples
Mesquita et al. 2011a, b	Optical light+epifluores-cence	×100, ×200	Bright Field	CCD Camera (150+100)	Matlab	Bright field image processing: (1) pretreatment (background removal) (2) Aggregates segmentation (3) Filaments segmentation (4) Debris elimination (5) Quantification and characterization Color image processing: (1) image pretreatment (background removal) (2) Aggregates segmentation (3) Filaments segmentation (4) Fluorescent-based intensity (5) Quantification and characterization

among others tasks. It can also be used to predict filamentous bulking (Banadda et al. 2005) and quantify morphological changes occurring in the biomass during special events in anaerobic reactors. An overview of image analysis applications in biological WWT processes is presented in "Fields of application."

Fields of application

One advantageous aspect of image processing that makes it an interesting topic of study is the diversity of its applications. Virtually, every branch of science has disciplines that use recording devices or sensors to collect image data from the universe around us. Furthermore, several software packages, such as ImageJ (National Institute of Health, USA), Matlab (The Mathworks, Inc., USA), Visilog (Noesis, France), and DAIME (University of Vienna, Austria), offer the possibility of processing the images to extract the relevant data.

Currently, in the biotechnology field, automated analysis covers the whole spectrum of microorganisms: bacteria, yeast, fungi, plant, and mammalian (human and animal) cells, protozoa, and metazoa, as well as cell components such as key morphological features, and even RNA fragments (Pons and Vivier 1999). Image processing and analysis has now gone beyond the stage of pure technical and mathematical development and can be used in bioresearch and production and product quality control. Relatively to its application to complement the well-established WWT operating parameters measurements, the use of image analysis is constantly growing. Both aerobic and anaerobic processes may profit from this novel technique, mainly on physiological and morphological characterization of microbial aggregates and evaluation of filamentous bacteria contents.

Aerobic wastewater treatment biological processes

The activated sludge system is the most significant aerobic WWT process. Typically, an activated sludge consists of a complex system composed of different types of microorganisms (bacteria, protozoa, metazoan, fungi, and algae). Furthermore, imbalances between the different types of bacteria may take place and disturb the plant. For this reason, microbial community assessment by microscopy inspection is useful in the rapid diagnosis of a problem (Madoni 1994a; Salvadó et al. 2004; Eikelboom 2000; Jenkins et al. 2003). An essential aspect of plant operation is the maintenance of activated sludge settling properties within an appropriate range. Sludge settling problems are largely related to the morphological and surface characteristics of flocs and to filamentous bacteria proliferation. These problems are generally identified using the sludge volume index (SVI), which is the most used parameter to characterize the sludge settling ability.

As reported by Sezgin et al. (1978), there seems to be a well-defined relationship between SVI and filamentous bacteria contents, thus opening the door for the application of quantitative image analysis in monitoring and control of aerobic WWT biological processes. Table 3 summarizes some key studies on the application of quantitative image analysis and the most significant morphological parameters in activated sludge processes. In fact, a succinct analysis of Table 3 indicates that the SVI parameter, as an indicator of biomass settling ability and filamentous bulking, is highly correlated with filament content, although no general equation has been widely accepted. It seems that quantitative image analysis is a powerful tool to detect process disturbances in activated sludge WWTP.

Problems of sludge settling ability

In activated sludge systems, an adequate balance between the different types of bacteria is necessary to ensure efficient removal of pollution, good sludge settling abilities, and low suspended solid levels in the final effluent (Mesquita et al. 2009a). Since the emergence of activated sludge systems for biological WWT almost a century ago, filamentous bulking has caused operational problems in plants worldwide. Sludge settling deterioration is commonly characterized by an imbalance between floc-forming and filamentous bacteria in the microbial aggregates of the sludge (Jenné et al. 2007).

The SVI parameter determined by the volume fraction of sludge after a certain settling time (usually 30 min) normalized by the TSS is the most used parameter to measure the sludge settling ability in a WWTP. However, usually the plant is already suffering from a filamentous bulking phenomenon when this parameter increases above a critical value, 150 mL/g. Grijspeerdt and Verstraete (1997) studied the use of morphological parameters to estimate settling ability of activated sludge, which was one of the first works concluding that image analysis parameters were more sensitive to changes in settling ability than the diluted SVI, suggesting that this data could be used as an early warning in reactor monitoring and control. In their work, both the form factor (FF; Table 1) and the D_{eq} started to change >5 days earlier than the diluted SVI, being the FF the parameter with most potential for monitoring because of its higher sensitivity. Therefore, the results indicate that a spherical and smooth surface is linked with better settle ability, since the FF parameter represents the deviation of an object to a circle (in this case the value measured should be 1), being special sensitive to the object roughness.

The relationship between SVI and total filament length (representing filamentous bacteria contents) was clearly

Table 3 Synopsis of quantitative image analysis applications in activated sludge monitoring

Reference	Plant	Main morphological parameters[a]	Correlations/observations[b]
Mesquita et al. 2011a, b	Lab-scale	21 parameters+Gram+/Gram− bacteria and viable/damaged	Bulking prediction (PCA[c])
Tian et al. 2011	Lab-scale	D_{eq}, TL/TA, Ff, AR, Ro	Filamentous bulking prediction
Van den Broeck et al. 2011	Full-scale	A_{mean}, %1 pixel	Filterability prediction
Arelli et al. 2009	Pilot-scale	TL, AR, FD	Relation between dSVI and the AR/TL ratio
Mesquita et al. 2009c	Full-scale	38 parameters	SVI prediction (PLS[c])
Yu et al. 2009	Full-scale	D_{eq}, FD, TA, TV	SS prediction (ANN[c])
Mesquita et al. 2009b	SBR	a_{Nb}, TA, %Area, %Nb, Sol, Ro, Ext, CF, Ecc, Conv, Rb	SVI prediction (PLS[c])
Mesquita et al. 2009a	Full-scale	36 parameters	SVI prediction (PLS[c])
Mamane et al. 2008	Pilot-scale	Nb, D_{eq}, FF, FerD	–
Pandolfi et al. 2007	SBR	Number of blue pixels	PHB storage in filaments
Smets et al. 2006	Lab-scale	AR, D_{eq}, FD, FF, Rg, Ro, TL	Best combination for modeling the SVI: TL+FD+(Ro, Rg, or AR)
Liao et al. 2006	SBR	Flocs size	↑SRT→↑Flocs Size; ↑SVI→↓Flocs size
Jenné et al. 2006	Lab-scale	AR, D_{eq}, FD, FF, Rg, Ro, TL	↑SVI→↑FD and ↑TL (PCA[c])
Alinsafi et al., 2006	Lab-scale	D_{eq}, DF, L_{fi}, fNb	Effects of dyes: ↓(D_{eq}, L_{fi}, fNb)
Amaral and Ferreira 2005	Full-scale	Conv, Ecc, Sol, TA, TL, TL/TA	↑SVI→↑ TL/TSS; ↑TSS→↑TA (PLS[c])
Banadda et al. 2005	Lab-scale	D_{eq}, FF, L_{fi}, Rg, Ro	↑(Rg, L_{fi}, D_{eq}, SVI) vs. ↓ (Ro, Ff)
Liwarska-Bizukojc and Bizukojc 2005	Lab-scale	A, D_{eq}, P, Conv, ferD	↑toxic→↓A
Pandolfi and Pons 2004	Lab-scale	Number of blue and red pixels	Gram-negative and Gram-positive bacteria determination
Casellas et al. 2004	SBR	A, fNb, L_{fi}	↑SVI→↑Lf and ↑fNb
Contreras et al. 2004	-	Rg, Ro	Filaments: Rg>1.06 or Ro<0.38
Jenné et al. 2003, 2004	Lab-scale	A, FF, fNb, L, L_{fi}, P, Rg, Ro	↑SVI→↑L_{fi}
da Motta et al. 2003b	Lab-scale	D_{eq}, FD, L_{fi}	↑SVI→↑L_{fi}
Wilén et al. 2003	Full-scale	D_{eq}, FD, fNb	
Jin et al. 2003	Full-scale	D_{eq}, FD, fNb	↓fNb→↑sludge settling
Jenné et al. 2002	–	AR, FD, FF, Rg, Ro	Rg most suitable to distinguish Filaments/flocs
Heine et al. 2002	Lab-scale	e_{Fil}, e_{flocs}	↑SVI→e_{fil} ; toxic inflow: ↑e_{flocs}
Govoreanu et al. 2002	SBR	CF, EF, HCF	–
Cenens et al. 2002	–	AR, FD, FF, Rg, Ro	Rg most suitable to distinguish Filaments/flocs
da Motta et al. 2001b	Full-scale	D_{eq}, FD, fNb, L_{fi}, TL/TA	↑L_{fi} before ↑SVI
Grijspeerdt and Verstraete 1997	–	AR, D_{eq}, FD, FF, Ro	↓Ff before ↑SVI
Ganczarczyk 1994	Full-scale	A, D_{eq}, a_L, P	↑v_{sed} → ↓D_{eq} and ↓L

[a] *%1pixel* surface fraction of activated sludge particles equal to 1 pixel, *%Area* total aggregates projected area distribution by equivalent diameter ranges, *%Nb* total number of aggregates distribution by equivalent diameter ranges, *A* aggregates area, a_L aggregates length, A_{mean} mean particle size, a_{Nb} aggregates number, *AR* aspect ratio, *CF* compactness factor, *Conv* convexity, D_{eq} aggregates equivalent diameter, *Ecc* eccentricity, e_{Fil} filamentous fraction, e_{flocs} flocs fraction, *EF* elongation factor, *Ext* extent, *FD* fractal dimensions, *ferD* Feret diameter, *Ff* form factor, *fNb* filaments number, *HCF* Heywood circularity factor, L_{fi} total filaments length per image, *P* aggregates perimeter, *Rg* reduced radius of gyration, *Ro* roundness, *Rb* robustness, *Sol* solidity, *TA* total aggregates area, *TL* total filaments length, *TL/TA* ratio between total filaments length and total aggregates area, *TV* total particles volume, *W* aggregates width

[b] *SVI* sludge volume index, *SRT* sludge retention time, *PHB* poly-β-hydroxybutyrate, *SS* suspended solids

[c] Correlations detected by multivariate statistical analysis: *ANN* artificial neural network, *PCA* principal components analysis, *PLS* partial least squares regression, *RBFNN* radial basis function neural network

demonstrated in the works of da Motta et al. (2001b, 2003a), Jenné et al. (2003), and Casellas et al. (2004) in full and laboratory scale and sequencing batch reactors (SBR), respectively. Figure 4a shows an example of a slugde without bulking problems, while Fig. 4b shows a picture from sludge with a bulking phenomenon, where is visible the high content of filamentous microorganisms. According to Jenné et al. (2004), a significant change in floc shape was observed when the bulking event occurred: the flocs roundness decreased and their elongation, represented by the Rg, increased. Furthermore, the total filament length and the floc boundary roughness described by the FF were selected as having the most potential for monitoring purposes. However, the predictive ability of the total filament length parameter is limited since it increases at the same time as the SVI (Banadda et al. 2005). Image analysis offers promising perspectives for early detection of filamentous bulking because the morphology parameters of the activated sludge respond quickly to changing process conditions (Smets et al. 2006). Therefore, the best performing models should have a combination of the total filaments length, one of the floc elongation parameters (roundness, Rg, or aspect ratio, AR; Table 1), and the fractal dimension (FD) as inputs. These results clearly show the need to couple all these parameters because alone they cannot predict the SVI increase. However, the total filament length is a valuable input in the model.

The impact of the floc structure on the sludge compressibility and settling ability was assessed through a series of statistical data analyses and expressed with significant relationships (Jin et al. 2003). The morphological and physical properties of the sludge flocs had relatively more significant influence on the compressibility and settling ability than its chemical properties. The floc size, fractal dimension, and filament index were the major parameters associated with SVI. Additionally, activated sludge containing relatively small and compact flocs with low numbers of filaments had better compressibility and settling ability properties. Jenkins et al. (2003) found that the SVI was strongly influenced by the FD and filament index of sludge flocs. In another study, activated sludge samples from seven full-scale plants were investigated to determine the relationship between floc structure and stability (Wilén et al. 2003). The floc macrostructure characteristics, floc size distribution, FD, and filament index, were the most important factors governing the stability, i.e., large and open flocs break up more easily than smaller and rounder ones when exposed to shear forces.

Sludge settling ability can also be correlated with microscopic parameters using chemometric techniques. A close correlation between the filamentous bacteria per suspended solids ratio and the SVI was achieved during filamentous bulking events using partial least-squares regression (PLS) (Amaral and Ferreira 2005). However, this relation was obtained only during severe bulking phenomena with high SVI values that limited its application. Therefore, a similar strategy was applied but with a broad range of SVI values (Mesquita et al. 2009a). The results unveiled a strong relationship between the sludge settling properties, as described by the SVI, and some morphological descriptor groups of filamentous and aggregated biomass, such as the free filamentous bacteria content, and aggregate size and morphology. This established relevant relationships between macroscopic and microscopic properties of the biological system. The relation between activated sludge image information and SVI measurement was also studied using principal component analysis (PCA) (Jenné et al. 2006), the most relevant set of sludge features would be made of the total filament length, an elongation measurement (e.g., Rg) and the FD.

A model that uses a chemometric tool to extract relevant data from the several morphological parameters is needed. From the several studies reported, bulking phenomenon seems that can be anticipated by a modification in flocs morphology, namely, a filaments release and a change in flocs roundness (less spherical and more elongated) and roughness (more irregular). All this can be detected by the morphological parameters TL, FF, Rg, and FD, which deserve a special attention. Although, in this case, the determination of this parameters seems difficult and time consuming than the usual SVI determination, the possibility to detect a bulking problem in an embryonic stage is very important because it allows to take preventive measures before the problem evolve into an irreversible stage. This represents the true advantage of use quantitative image analysis to detect sludge settling ability problems compared with traditional methods.

Sludge contents

The use of image analysis to estimate sludge concentration ($X_{biomass}$) was attempted based on the assumption that an increase in sludge concentration leads to an increase of the projected area of the sludge. The first linear relation between the quantitative image analysis parameter, field area (the area percentage in an image), and the $X_{biomass}$ was found by Grijspeerdt and Verstraete (1997). However, this calibration cannot be applied to highly concentrated sludge (>4 g L^{-1}), where a dilution is needed. This calibration depends on the sludge type and density, and therefore, it should be carefully applied. A linear correlation between TSS and mean projected area was also found within the study of biodegradation processes with and without sodium dodecyl sulfate (SDS) (Liwarska-Bizukojc and Bizukojc 2005).

Amaral and Ferreira (2005) used multivariate statistical PLS regression to elucidate the morphological parameter relationship with TSS on a full-scale plant. They studied

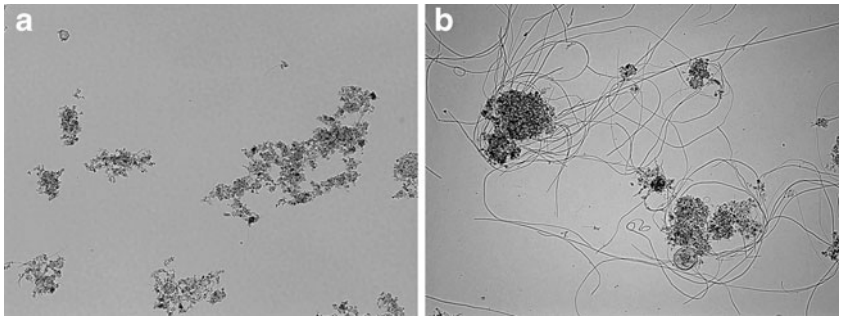

Fig. 4 Bright field images of normal flocs (**a**) and filamentous bulking (**b**) from a lab-scale activated sludge system acquired in an Olympus BX51 optical microscope (Olympus, Tokyo, Japan) with ×100 total magnification

three key aspects, free filamentous bacteria contents, aggregate contents, and aggregate morphology. The key aspect of aggregate contents, namely the total aggregate area (TA), was considered as the parameter that most contributes to estimate the TSS.

An important aspect regarding the prediction of sludge concentration by the morphological parameter total area is related to the nonlinear characteristic of this correlation because the particles densities are not constant. To overcome this problem, an artificial neural network (ANN) model was created (Yu et al. 2009), which was very effective in representing relationships in non-linear systems. They obtained a correlation with an R^2 of 0.932 between the measured and predicted suspended solids concentration. It seems undeniable the need to couple the morphological parameters with a chemometric technique to properly measure biomass concentration. This will be especially suitable when applied in an online expert system.

Toxic compounds

The early detection of toxic compounds is an advantage of use morphological parameters to monitor and control the efficiency of activated sludge processes, although the morphological parameters cannot identify the type of toxic. When the toxic concentration did not exceed high values, the detection of toxics is possible because a significant change in the flocs morphology will occur before the operational parameters change. When the concentration reaches high levels, the inhibition may be almost immediate.

Heine et al. (2002) found that the inflow of a toxic substance (phenol) could be detected immediately by the image analysis procedure causing an increase in the fraction of microflocs ($e_{\text{microflocs}}$; Table 1). Such an early forecast is impossible with classical physicochemical parameters, leading to irreversible damage due to the time it takes for the problem to be detected.

Another toxic compound of activated sludge systems are surfactants. Liwarska-Bizukojc and Bizukojc (2005) developed an image analysis procedure to measure the morphological parameters of activated sludge flocs in contact with SDS. The floc mean projected area decreased by ~30 % at SDS from 2.5 to 25 mg L^{-1} and ~60 % at from 250 to 2500 mg L^{-1}. In addition, a sudden increase in filaments caused by defloculation was reported in a study on the characteristics of activated sludge exposed to textile dyeing wastewater (Alinsafi et al. 2006).

The effect of toxic substances on the mobility of a protozoon species, commonly found in WWTP, was also studied. The protozoan *Tetrahymena pyriformis* was exposed to four different toxic compounds (copper, zinc, cycloheximide, and Triton-X), leading to decreased motility and increased tumbling behavior (Amaral 1998; Amaral et al. 1998).

Biomass physiology

Pandolfi and Pons (2004) were pioneers in terms of coupling quantitative image analysis protocols with colored images, namely, to identify the Gram characteristic of protruding filamentous bacteria in an activated sludge ecosystem, and to characterize poly-β-hydroxybutyrate (PHB) storage in filamentous bacteria (Pandolfi et al. 2007). These authors developed an automated image analysis method taking a pixel as "blue" when its blue level (B) on the blue primitive of as RGB image is larger than its red level on the red primitive (R). Inversely a pixel was considered "red" when R was >B. Images with >50 % "blue" pixels were considered Gram negative (Pandolfi and Pons, 2004), and the PHB content was assessed by the percentage of "blue" pixels within the filaments (Pandolfi et al. 2007).

However, the use of classical Gram-staining methodologies (such as the Hucker method) may lead to large and dense flocs not decolorizing correctly, thus becoming a problem in identifying Gram status through image analysis procedures. Furthermore, the development of novel fluorescent nucleic acid binding dyes allows the assessment of Gram status by differential absorption through bacterial cell walls and without fixation. As a result, the use of these fluorescent dyes can provide a robust, objective, and rapid alternative to traditional Gram-staining in wastewater systems (Foster et al. 2002).

For viability detection, fluorescent nucleic acid binding dyes that differ in their ability to penetrate healthy bacterial cells can differentiate live from dead or damaged cells by membrane integrity, even in a mixed population containing

a broad range of bacterial types (Invitrogen Molecular Probes 2004 This kit was successfully applied to drinking water and activated sludge samples using epifluorescence microscopy (Boulos et al. 1999; Lopez et al. 2005; Louvet et al. 2010) by the development of specialized image analysis procedures (Hug et al. 2005; Lopez et al. 2005; Zhou et al. 2007). Amaral et al. (2010a) emphasize the importance of the use of fluorescent nucleic acid binding dyes to determine the filamentous bacteria Gram and viability status in WWT monitoring.

A program in Matlab (The Mathworks, Inc., Natick, USA) was developed to recognize and characterize aggregated and filamentous biomass physiology by fluorescence microscopy (Mesquita et al. 2011a). For this purpose, SYTO 9 and PI stains (Invitrogen Molecular Probes 2004) were used in viability assessment and SYTO 9 and HI (Invitrogen Molecular Probes 2011) for Gram status determination. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain, 490/635 nm for PI, and 480 nm/625 nm for HI. This methodology required the extraction of the green channel from the original RGB image collected with a green filter (excitation bandpass of 470–490 nm and emission above the cut-off value of 516 nm) and with a red filter (excitation bandpass of 530–550 nm and emission above the cut-off value at 591 nm). For each of the above channels, the program performed a background correction, image segmentation, and aggregated and filamentous biomass recognition. The developed program also allowed for the calculation of a fluorescence-based intensity image for both green and red channels to be directly correlated with the fluorescence of the aggregated and filamentous biomass. These images were subsequently used to identify Gram and viability status (Fig. 5).

For an in-depth microbial structure characterization, the CLSM may be useful because it provides a better resolution compared to epifluorescence microscopy. Nowadays, CLSM is widely employed in studies with activated sludge, especially to visualize and quantify FISH probed populations (Schmid et al. 2003; Schuppler et al. 1998a, b; Larsen et al. 2008; Davenport et al. 2000). Other characteristics, such as the floc interior structure, can also be studied with this technology (Chung and Lee 2003; Chu et al. 2005), thus contributing to the development of specialized software for CLSM images (Daims et al. 2006; Lopez et al. 2005; Daims and Wagner 2007).

Furthermore, identification of phosphate (PAO)- and glycogen (GAO)-accumulating organisms by inclusions staining and FISH techniques has been attempted by Carvalho et al. (2007) on the microbial characterization by FISH analysis of denitrifying phosphorus removal systems, Serafim et al. (2002) on staining procedures for visualizing intracellular polymers and FISH analysis for PAO identification, and Levantesi et al. (2002) for the structure of bacterial aggregates and identification PAO (by FISH) of polyphosphate and poly-β-hydroxyalcanoate (PHA) inclusions.

Full-scale WWT plants

There are already a few published works that have attempted to monitor full-scale WWTP. Da Motta et al. (2002a, 2003a) monitored a total of 12 different WWTPs in France and Portugal, emphasizing the importance of the aggregates D_{eq}, total filaments length (TL), and filaments number (fNb) for detecting activated sludge disturbances. The works of Amaral (2003) and Amaral and Ferreira (2005) further elucidated the relationship between SVI and TSS with key image analysis data by monitoring a WWTP with filamentous bulking over 3 1/2 months. These authors introduced a segregated morphological analysis by floc size and showed the importance of the total filament length per total suspended solids ratio (TL/TSS; Table 1) for filamentous bulking recognition and monitoring. Furthermore, Mesquita et al. (2009c) predicted the SVI in normal operating conditions and filamentous bulking by using image analysis data coupled with PLS techniques. Finally, Amaral et al. (2010a, b) extended the identification of activated sludge problems to viscous bulking, filamentous bulking and pinpoint floc formation. Currently, development of online systems to monitor full-scale WWTP is a challenge that when implemented could dramatically change the usefulness of quantitative image analysis techniques. The sampling method and images acquisition are the main barriers, which need further investigation and development.

The identification of protozoa and metazoa populations, which are among the most important indicators of activated sludge status (Madoni, 1994b), is another important field of application of image analysis. Coupled with multivariate statistical techniques, such as PCA (Amaral et al. 1999; da Motta et al. 2001a) and discriminant analysis (DA) (Amaral et al. 2004a), morphological parameters can be used to recognize >80 % of the main 22 protozoa and metazoa species commonly present in aerated tanks. Ginoris et al. (2007a, b) further corroborated the usefulness of DA and ANN for identification purposes as well as the possibility of real-time analysis (2–3 h), while avoiding the tedious task of traditional manual identification.

These microorganisms are characterized by high grade of locomotion and motility; therefore, the acquisition of sharp images can be very difficult. As stated in "Operation mode," the rapid technological developments in the last years allow us to use cameras capable of aquire images at ultra high shutter speed. The use of two opposed lenses and mounting the sample between two coverslips and the use of flashs and maybe backlight may represent a solution to overcome this problem. Neverthless, the increase in the equipment costs will be substantial.

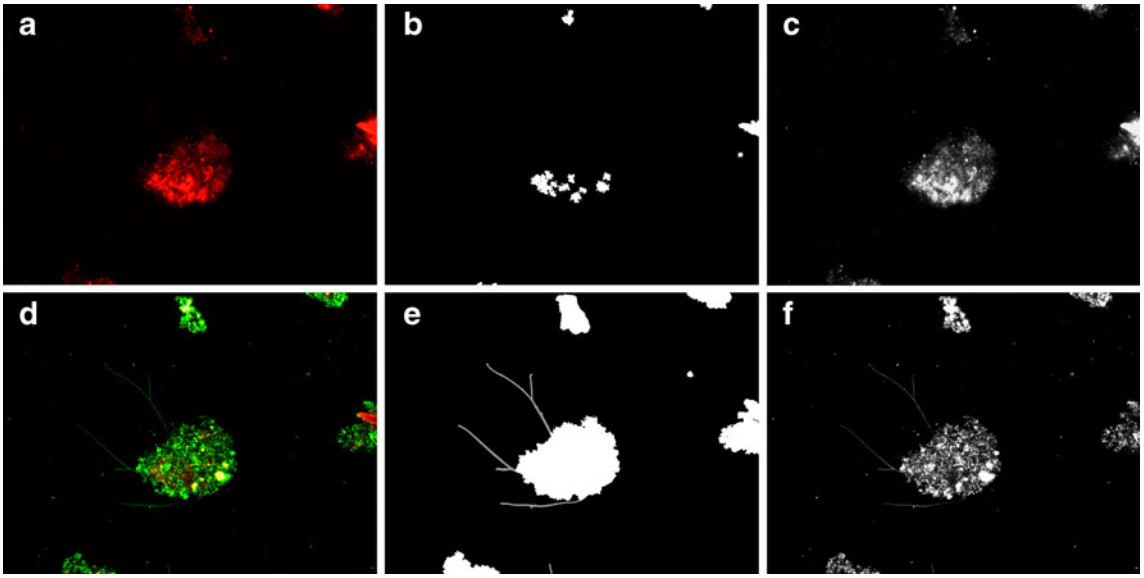

Fig. 5 Biomass from a lab-scale activated slude system dyed with SYTO 9 and propidium iodide, taken with a green filter, HIW, 20 nm (**a**–c) and a red filter, HIW, 20 nm (**d**–**f**). Original images acquired in an Olympus BX51 optical microscope (Olympus, Tokyo, Japan) with ×200 total magnification (**a**, **d**), area detection images (**b**, **e**), intensity images (**c**, **f**)

Aerobic granulation

Development of aerobic granules is gaining interest during the last years. They are mainly cultivated in SBR and airlift reactors (Beun et al. 2002). The possibility to operate high-rate reactors based on granular sludge has several advantages, such as a denser and stronger aggregate structure, better settle ability and ensured solid-effluent separation, higher biomass concentration, and greater ability to withstand shock loadings (Liu et al. 2009a, b). In addition, less footprint is needed in full-scale applications.

Image analysis techniques have been applied to better understand the granules morphology. Beun et al. (2002) determined the granules D_{eq}, shape factor, and AR to follow the granulation process in a sequential batch airlift reactor. It was found that the AR and shape factor stabilized (around 0.7–0.8) earlier than the average D_{eq}. In addition, particle size distribution curves were used to detect granulation stability.

Image analysis was applied to follow the granulation process of aerobic granules in a SBR treating soybean processing wastewater, by using an optical microscope to determine the D_{eq}, AR, and roughness. The formation of granules was a gradual process, demonstrated by the increase in mean diameter from 0.10±0.05 to 1.22± 0.85 mm. It was also found that the matured granules had a FD of 1.87±0.34. The Ro and AR decreased to 1 during the granulation process confirming that aerobic granules are dense, regular, and round aggregates (Su and Yu 2005).

One of the problems associated with granules regards the mass transfer limitation caused by its denser structure. Image analysis using CLSM coupled with staining techniques can be used in mathematical models to evaluate mass transfer and identify the distribution of EPS and cells inside aerobic granules. The internal structure of aerobic granules was already explored using nitrogen adsorption method and CLSM technique. It was found that the granules had a complex structure of cell clusters, discrete aggregates of cells in an EPS matrix and many interstitial voids (Liu et al. 2010).

Anaerobic wastewater treatment biological processes

During the last decades with the increasing use of anaerobic granular sludge in wastewater treatment processes, the study of structural changes in terms of size, strength, density, and settling ability of anaerobic microbial aggregates is accelerating. Several direct and indirect methods to quantify the physical characteristics of the anaerobic granular sludge have been presented during the last decades (Ahn and Speece 2003).

Since Jeison and Chamy (1998) presented a technique for measuring the size distribution of granules using a scanner to acquire digitalized images of samples embedded in gelatine, ample specialized programmes/protocols have become available to measure in a straightforward manner the morphological characteristics of the biomass. In the field of anaerobic WWT biological technology, works reporting the use of quantitative image analysis (Table 4) range from simple differentiation between flocs and granules (Bellouti et al. 1997) to the characterization of hydrogen-producing granules (Mu and Yu 2006b).

Anaerobic granulation phenomenon

During initial experiments with an anaerobic filter, Lettinga (2001) found that, in addition to attached biomass, a large portion of the sludge aggregated to form granules within the interstitial voids of the support media. The recognition of the sludge granulation concept was a significant mark in anaerobic WWT, which has greatly enhanced both the efficiency and applicability of this technology. Consequently, the recognition of the granulation phenomenon has always been a topic of interest for quantitative image analysis research. The first attempts to use digital image analysis in anaerobic WWT processes were limited to size measurements and number counting (Dudley et al. 1993). In this work, the number of granules correlated well with dry weight determinations (R^2= 0.989). The authors were able to determine an increase in the granule size with time throughout the digestion process, supported by dry weight determinations indicating an increase in biomass. Singh and Viraraghavan (1998) were among the first authors to report the use of image analysis to reveal the sludge aggregation status by measuring granule diameter and number. No surprise in the findings that the granules diameter increased and the number decreased along the granulation process. Couple to this morphological change a decrease in the SVI was observed.

A morphological parameter based on the ratio of the specific total filament length to the total aggregate projected area (*L*fA, Table 1) allowed the recognition of the aggregation time and the monitoring of biomass washout (Araya-Kroff et al., 2004). Other measurements, such as the percentage of projected area of aggregates within 3 D_{eq} size classes (0.01–0.1 mm, 0.1–1 mm, and >1 mm) and the volatile suspended solids per total aggregates projected area (VSS/TA; Table 1), reflected the aggregation/granules maturation phenomena. The results showed quantitatively the dynamics of structural and morphological sludge during the process of granulation (Fig. 6). Furthermore, it demonstrated the usefulness of the proposed parameters in describing the occurrence of filaments release and aggregate fragmentation resulting from granule erosion induced by changes in the up-flow velocity and of the organic loading rate.

Quantitative image analysis may provide useful information to understand the behavior of anaerobic granules either at research and full-scale level. It is important to understand the mechanisms of granulation and development of granulation theories, but it can also be valuable to timely detect potential problems with the granules stability. In this case, the implementation of an online monitoring system may be very advantageous compared to the classical offline measurements.

Toxic events

In the last few decades, high-rate anaerobic reactors, based on granular sludge, have become widespread due to their high efficiency and stability. However, restocking granules to substitute those lost by excessive granular sludge breakdown and consequent washout is a common problem in these reactors. Since granular sludge is expensive, its stability and maintenance is a sensitive issue that justifies appropriate attention and study.

The deterioration of granular sludge induced by prolonged contact with oleic acid was studied by quantifying the percentage of aggregates with D_{eq}<1 mm both in terms of projected area and number of aggregates (Amaral et al. 2004b). When oleic acid concentration was increased, *L*fA was sensitive to the sludge deterioration process and indicated, with the anticipation of approximately 1 month, the most significant biomass washout episode that occurred in the trial period. The authors proposed a mechanism of filaments release, detachment and selective washout to explain the effect on *L*fA.

In a different experiment, a commercial detergent and a solvent were fed to lab-scale expanded granular sludge bed (EGSB) reactors to detect the effects of toxic compounds from the cleaning stages (Costa et al. 2007, 2009b). The inhibitory effects were dependent on the surfactant concentration and exposure time. Furthermore, the *L*fA parameter acted as an early-warning indicator of the biomass washout, since it increased 3 and 5 days before effluent VSS increase, respectively, in shock with 150 and 300 mg COD L^{-1} of detergent (Costa et al. 2007). During the solvent shock load (Costa et al. 2009b), the percentage of the aggregates projected area with D_{eq}>1 mm decreased from 81 to 53 %. Furthermore, the mean D_{eq} of the aggregates >0.2 mm decreased, as well as the settling velocity, showing that the granules underwent a fragmentation process (Fig. 7a, b), and consequent filament release (Fig. 7c, d). Once again, the *L*fA parameter increased 2 days before the effluent VSS, suggesting that the *L*fA was an early-warning indicator of washout events during toxic contamination.

The possibility to measure the protruding filaments that are still connected to the granules before its release and possible granules fragmentation is one of the main advantages of quantitative image analysis. In this way, it is possible to detect potential problems before they develop to irreversible states.

Organic overloads

Four organic loading disturbances were performed in lab-scale EGSB reactors fed with ethanol (Costa et al. 2009a). Granule erosion/fragmentation was observed after increase the organic loading rate (OLR) from 5 to 18.5 kg COD m^{-3} day^{-1}, given the decrease from 90 to 70 % of the percentage of aggregate projected area with D_{eq}>1 mm. An immediate granule erosion/fragmentation was also observed after increasing the OLR to 50 kg COD m^{-3} day^{-1}

Table 4 Synopsis of quantitative image analysis applications in anaerobic wastewater treatment processes

References	Reactor[a]	Role of image analysis	Morphological parameters[b]	Correlations/observations
Abreu et al. 2011	EGSB	Structural changes of granules producing H_2	%Area, TL/VSS, VSS/TA	
Costa et al. 2009a	EGSB	Structural changes during organic loading disturbances	%Area, *L*fA, TL/VSS, VSS/TA	
Costa et al. 2009b	EGSB	Structural changes during solvent shock load	%Area, *L*fA, TL/VSS, VSS/TA	
Costa et al. 2007	EGSB	Structural changes during detergent shock load	%Area, *L*fA, TL/VSS, VSS/TA	
Abreu et al. 2007	EGSB	Structural changes during SAA recovery	%Area, *L*fA, TL/VSS, VSS/TA	Onset biogas production with ↑TL/VSS
Mu and Yu 2006a	UASB	Fractal dimensions of granular sludge	FD	FD=2.79±0.03, rheological vs. *df* characteristics
Mu and Yu 2006b	UASB	Morphology of H_2-producing granules	%Nb, D_{eq}, FD	d=1.0–3.5 mm, FD=1.78
Amaral et al. 2004b	EGSB	Oleic acid effects on biomass morphology	%Area, %Nb, *L*fA	*L*fA as early-warning of washout
Araya-Kroff et al. 2004	EGSB	Quantify granulation process	%Area, D_{eq}, *L*fA, TL/VSS, VSS/TA	*L*fA to detect granulation time
Singh and Viraraghavan 2003	UASB	Impact of temperature on granulation	D_{eq}, FD	↓T→↑D_{eq}
Pereira et al. 2003	EGSB	Effects of ↑[oleic acid] in morphology	D_{eq}	→[oleic acid]□↓D_{eq}
Ahn and Speece 2003	UASB	Validate settleability protocol	%Nb	↑ vs→↓v_{sed} and D_{eq}
Alves et al. 2000	UFBR	Filaments changes during organic shocks	freefNb, TL	fNb and TL ↑→SAA ↑ (low [substrate])
Jeison and Chamy 1998	-	Granules size distribution	%Area	–
Singh and Viraraghavan 1998	UASB	Granulation during star-up at 20 °C	D_{eq}, a_{Nb}	↓HRT→↑d; ↓Nb→aggregation
Bellouti et al. 1997	UFBR	Flocs vs. granules	FD (box counting/power law)	FD (flocs)=1.90±0.02/1.84±0.13
	UASB			FD (granules)=1.95±0.01/2.14±0.08
Dudley et al. 1993	UASB	Characterize anaerobic granules	Number, size, and density	–

[a] *EGSB* expanded granular sludge bed, *UASB* up-flow anaerobic sludge blanket, *UFBR* up-flow anaerobic fixed bed reactor

[b] *%Area* total aggregates projected area distribution by equivalent diameter ranges, *%Nb* total number of aggregates distribution by equivalent diameter ranges; a_{Nb} number of aggregates, D_{eq} equivalent diameter, *FD* fractal dimension, *freefNb* free filaments number, *L*fA total filament length per total aggregates projected area, *TL* total filaments length, *TL/VSS* total filament length per volatile suspended solids, *VSS/TA* volatile suspended solids per total aggregates projected area

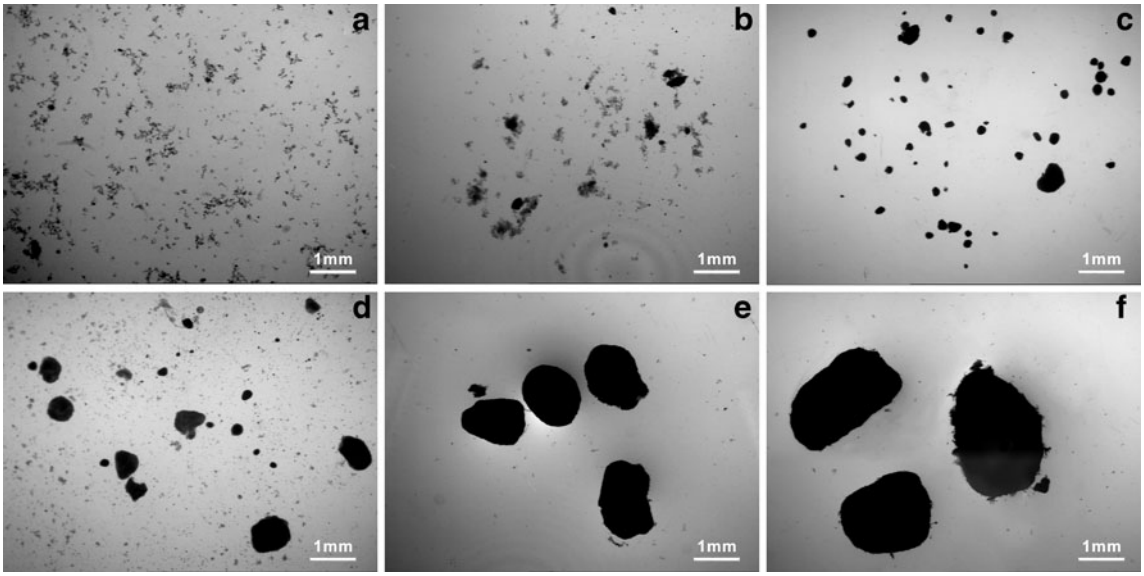

Fig. 6 Bright field images of aggregates, acquired through visualization on a SZ 40 stereo microscope (Olympus, Tokyo) with ×15 magnification, during an anaerobic granulation assay in a lab-scale UASB reactor

over 3 and 16 days, quantified by a reduction of 45 % in the percentage of aggregate projected area with D_{eq}>1 mm. The authors concluded that, in general, the selective washout of filamentous microorganisms was associated with granule erosion/fragmentation and a decrease in the specific acetoclastic activity (SAA).

Biomass activity

Important information can be obtained by combining morphological parameters obtained by quantitative image analysis techniques and physiological characteristics of microorganisms. In an anaerobic digester fed with synthetic substrate, with 50 % (as COD) oleic acid, organic and hydraulic shocks were performed by increasing stepwise the substrate concentration and reducing the hydraulic retention time (Alves et al., 2000). In both cases, the organic loading rate changed from 6 to 30 kg COD m^{-3} day^{-1}. The hydraulic shock induced a fast decrease in the number of free filaments and in the total filament length. During the hydraulic shock, the methanogenic acetoclastic activity was directly correlated with the number and total length of free filaments, suggesting that

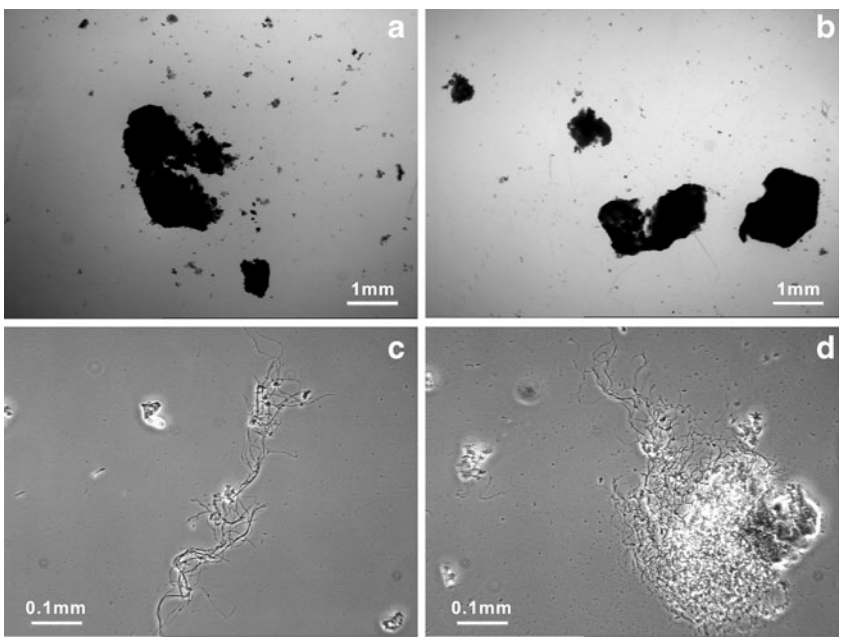

Fig. 7 Bright field images of granule fragmentation (**a**, **b**) and consequent release of filaments (**c**, **d**) during a solvent shock load in a lab-scale EGSB reactor. Granules images (**a**, **b**) acquired on an Olympus SZ40 stereo microscope (Olympus, Tokyo, Japan) with ×15 magnification, and flocs images (**c**, **d**) acquired on a Diaphot 300 microscope (Nikon Corporation, Tokyo, Japan) with ×100 magnification

these filaments were predominantly acetoclastic bacteria, probably *Methanosaeta*. In the organic shock, where levels of acetate in the effluent were 10-fold higher than after hydraulic shock, the acetoclastic activity was not correlated with the number and total length of free filaments.

Due to unspecified operational problems, the SAA of an anaerobic granular sludge present in an industrial up-flow anaerobic sludge blanket reactor was considerably hindered (from 250 to <10 mL $CH_{4@STP}g^{-1}$ VSS day^{-1}), thus significantly reducing the biogas production of the industrial unit. During the acetoclastic activity recovery test, the onset of SAA recovery, granule breakdown, and filament release to the bulk occurred simultaneously. Further increase in SAA was accompanied by granule size growth. In the last 25 days of operation, the size distribution was stable with >80 % of aggregate projected area corresponding to granules with D_{eq} >1 mm. Confocal images from FISH-hybridized sections of the granules showed that, after SAA recovery, the granules developed an organized structure where an acidogenic/acetogenic external layer was apparent (Abreu et al. 2007). Further microscopy coupled with molecular techniques has already been cited, such as the work of (Howgrave-Graham and Wallis 1993) using transmission electron microscopy to study the inner structure of aggregates. Furthermore, Ahn (2000) refer to the use of methanogenic bacteria autofluorescence as a further approach.

High-rate anaerobic reactors

It is equally feasible to obtain values of parameters measured in solid, liquid, or gaseous phases. For automatic monitoring and control, parameters in the solid phase are not often used, since they usually need tedious and difficult manual operations. So far, parameters involved in reactor control had been limited to indicators of the liquid and gaseous phases. The parameters most widely used in the liquid phase are the pH, volatile fatty acids, alkalinity, and COD concentrations, whereas in the gaseous phase, they are the CO_2, CH_4, and H_2 contents and gas production (van Lier et al. 2001). In this framework, quantitative image analysis techniques have emerged as promising tools to overcome these difficulties, providing quantitative parameters of the solid-phase evolution. Its use, coupled with multivariate statistical techniques, represents a significant advance in the monitoring and control of the granule morphology stability and helps to define parameters to detect in real time problematic situations. Quantitative image analysis techniques have already been used to describe micro- and macrostructural changes of anaerobic granular sludge during transient periods, organic load disturbances (Costa et al. 2009a), and toxic load shocks (Costa et al. 2007, 2009b). Later, the application of PCA to these datasets, obtained during the transient states, determined the latent variables, encompassing a weighted sum of performance, physiological, and morphological information. These new variables were highly sensitive to reactor efficiency deterioration, showing remarkable variation in the first hours of the disturbances. The high loadings associated with image analysis parameters showed that the solid phase (biomass) morphological changes should be considered of interest to monitor and control load disturbances and toxic events in high rate anaerobic (granular) sludge bed digesters (Costa et al. 2009c, 2010).

Other applications

Particle sedimentation studies are another important field of quantitative image analysis application because particle size distribution of the suspended particles is one of the major parameter for solid–liquid separation efficiency. A high magnification microlens was applied to replace the microscope, combined with a high-resolution CCD to acquire images that can be used for on-line measurements (Yu et al. 2009). A flow cell with a magnetic pump provided continuous samples. By combining the image analysis data and an ANN model, this method could predict the suspended solids concentration and the particle settling efficiency of samples. Therefore, it is an image analysis technique that could substitute a laser particle size analyzer, which is expensive and difficult to use for on-line measurements (Williams et al. 2007). The in situ recognition is shown to be a powerful tool for performing real-time, in situ particle imaging acquisition to determine floc-size distribution during flocculation, expressed as temporal changes in floc average size (He et al. 2012).

The pore size distribution (PSD) of porous media can also be obtained using methods based on image analysis. An image-based method to measure PSD of general porous structures using CLSM images to reconstruct a 3D layer was developed to study the influence of the internal organization on the transport processes inside porous structures (Yang et al. 2009). According to the authors, the minimum pore size detected by the method equals to the physical size of pixels in the image; therefore, the accuracy of the method is linked with the image resolution.

Application of image analysis to monitor color in water and wastewater fields has been applied for several purposes, such as online monitoring of wastewater color (Yu et al. 2005). To overcome the problem of different sample hues, a back-propagation neural network was successfully applied.

Currently, a great interest has aroused in biotechnological applications, in the development of quantitative digital image analysis techniques to stained and fluorescent samples. Usually, this analysis is coupled with multivariate statistical analysis, such as PCA, ANN, cluster analysis (CA), PLS, or DA. An automated quantitative FISH method for complex environmental samples such as manure and soil was developed

in order to reduce interference from cell aggregates (Zhou et al. 2007). According to the authors, the use of CA, i.e., distinguishing groups within a data set as a way of handling this variability, solves the limitations of threshold-based classification methods due to nonuniform background, as well as the intensity variation of target and nontarget cell signals within an image and between experiments.

As stated in "Digital camera," LSM and MRI are being used to study complex microbial biofilms (another large field of application of quantitative image analysis techniques). All the LSM applications described so far record the intensity of fluorescence signals. However, the fluorescence signal contains two pieces of information. In addition to the intensity, the lifetime of an excited fluorochrome can be measured in a technique known as fluorescence lifetime imaging (Neu et al. 2010). Specially developed programs now incorporate procedures to automatically determine threshold values for 3D CLSM image stacks. Examples of such software packages are Auto PHLIP-ML (Merod et al. 2007), COMSTAT (Heydorn et al. 2000), ISA3D (Beyenal et al. 2004), DAIME (Daims et al. 2006), or PHLIP (Mueller et al. 2006), which calculate biofilm architectural metrics, including biovolume, mean thickness, roughness, percent area coverage, porosity, area/volume ratio, spatial spreading, and FD.

CLSM has some limitations that may be overcome by 2PLSM. Excitation with two photons of half the energy (usually infrared) allows a deeper penetration into lightscattering samples. Because of the longer wave length, two-photon imaging has an improved resolution in deeper sample locations. Another advantage is that the two-photon effect occurs only in the focal plane, thereby protecting outof-focus areas from bleaching (Neu and Lawrence 2005).

The popularity of LSM is based on the current broad availability of LSM instruments, the flexibility in terms of sample mounting and staining, as well as the option for quantitative analysis of digital data. Otherwise, MRI is not as readily available and requires more instrumental experience for measuring microbial samples and subsequent data analysis. This topic is in constant evolution, and new methods and techniques are being developed (Neu et al. 2010). CARD-FISH combined with CLSM is being ever more applied because it allows the maintenance of the biofilm structure while there characterization is performed (Lupini et al. 2011).

The presence of EPS can also be efficiently monitored by traditional epifluorescent microscopy and CLSM (Strathmann et al. 2002). In this way, the disruption of structures necessary in traditional biochemical analysis is avoided. Biofilm image analysis has not been completely automated, but new methods of automation and improvements to the current stages of automation are continually being made (Daims et al. 2006; Mueller et al. 2006).

Current state synopsis and future trends

The use of quantitative image analysis in monitoring activated sludge processes is well recognized, and some important correlations have already been established. The main problems relate to sludge contents and settling ability. Important relations were determined between the SVI parameter (the most used parameter to estimate sludge settling ability) and total filament length, floc elongation, and FD. However, no general equation has been generally accepted. Although, in this case, image analyses techniques may be more time consuming and difficult to implement than classical methods to determine SVI, they allow the recognition of potential bulking phenomenons in an earlier stage. Regarding to the sludge contents, some attempts have been made to correlate it with the total aggregate area detected by image analysis. Since these correlations are highly dependent of the sludge type, it might be a very difficult task to obtain a general equation. However, the use of chemometric techniques and expert systems to deal with the morphological parameters is being attempted with promising results in monitoring sludge contents, either in terms of biomass and VS concentrations.

Relatively to anaerobic digestion WWT processes, quantitative image analysis is mainly used as a tool to detect biomass morphological changes when sudden changes in the reactor's normal operation occur. Filament length, aggregate area and D_{eq} are the main indicators used in this field of application to detect in timely fashion granulation time, toxic contamination, or organic overload. The use of morphological descriptors, analyses of the reactor's performance, and microorganism's physiological information coupled with chemometric tools seem a promising strategy to detect operational problems before they reach an irreversible state.

Studies in real WWTP should be performed, especially in anaerobic digestion processes where there is a clear lack of studies on the use of quantitative image analysis. Indeed, the uncertainty adjacent to real industry effluents is the ideal scenario to confirm the usefulness and test the response of these techniques to the wide variations in effluent flow rate and composition usually found. In this way, it can distinguish between noisy variations and truly problematic situations.

The development of online quantitative image analysis systems for real-time process monitoring and control is one of the most important aspects in this field and is its ultimate goal. It may represent a significant and decisive milestone in the implementation of these procedures in wastewater treatment biological processes. Classical methods are time consuming and impossible to be implemented online. One of the main advantageous of image analysis procedures compared with classical methods is the possibility to implement an online system that can overcome the analyzer dependency and

time consuming of offline systems. However, there are some obstacles that need to be overcome before this can be achieved. The main obstacle for this implementation is not an image analysis protocol itself, but mainly to the technological scale-up difficulties for effectively obtaining undamaged, homogeneous, and representative samples from real WWTP. The first difficulty is to obtain a sample, which is representative of the entire WWTP. In laboratory prototypes, this is not much problem; however, given the dimensions and possible operational problems in full-scale WWTP, this objective is not easy to achieve. It will be necessary to define various strategic sampling points. Then, the sample should be homogenized without damaging the integrity and stability of flocs and/or granules; this is the most critical point of the whole process because it could lead to erroneous results. Therefore, the development of a sampling procedure that does not damage the biomass integrity and allow us to acquire sharp and useful images remains a challenge. Nevertheless, the problem of sharp images can be bypassed by increasing the shutter speed available in the cameras used or by adding flashs.

Another field where great effort is being made is in coupling quantitative image analysis with the microscopy of stained samples. The use of color scales as a substitute for brightness values allows the detection of small changes locally, improving characterization of the microbial community. In this way, it is possible to use these techniques together with Gram and Neisser staining, FISH, or other staining protocols. Such would be the case for monitoring the biomass present in SBR for wastewater treatment, in gaining further information on the biomass aggregation status, viability, and contents in real time. Dying techniques using fluorochromes and FISH would be advantageous for morphological characterization, viability, composition, and for quantifying polyphosphates, glycogen, and PHA inclusions.

Coupling morphological parameters obtained by online quantitative image analysis protocols with operational parameters in an expert system seems the most promising approach to properly monitor these processes, specially activated sludge and aerobic and anaerobic granular systems, and prevent potential damages. The possibility to detect these potential problems in an early stage, before they became irreversible, is the main advantage of quantitative image analysis over classical methods.

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